EXHIBIT 10
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STARCH FRACTIONS

[37] Iodimetric Determination of Amylose

Potentiometric Titration: Standard Method

By Thomas J. Schoch

George M. Moffett Research Laboratories,
Corn Products Co., Argo, Illinois

Introduction

Most starches contain two polysaccharide components: the linear fraction or amylose which adsorbs iodine strongly to give a deep blue complex, and the branched fraction or amylopectin which has only a weak affinity for iodine and gives a red coloration. Determination of linear fraction in starchy materials is accomplished by either of four general techniques: (a) potentiometric titration of the dissolved starch with standard iodine, (b) similar amperometric titration (Vol. IV [39]), (c) spectrophotometric determination of intensity of blue coloration with iodine (Vol. IV [40]), (d) sorption of Congo red (Vol. IV [41]).

The following potentiometric titration procedure represents the original method of Bates and co-workers (*1*) as modified by Schoch (*2*, *3*). It is applicable to all unmodified starches, the starch fractions, and various granular thin-boiling starches prepared by mild acid hydrolysis (that is, up to commercial 75-fluidity products). It is not applicable to more highly hydrolyzed products, torrefaction or enzymatic dextrins, oxidized starches, or starch derivatives. The calcium chloride method (Vol. IV [38]) should be used for the latter materials.

Procedure

Special Reagents

Stock iodine.—In a 2-liter volumetric flask, 166 g. of potassium iodide, exactly 4.000 g. of iodine, and 74 g. of potassium chloride are dissolved in a small amount of water. When the iodine has completely dissolved, the solution is diluted to the mark to give a solution of 0.5N with respect to potassium iodide, 0.5N in potassium chloride, and containing 2.000 mg. of iodine per ml. This solution is quite stable if stored in a glass-stoppered brown bottle away from light. However, it should be checked frequently by titration against standard sodium arsenite solution, and calculations should be corrected accordingly.

Iodine solution for titration.—The above stock solution is diluted exactly ten-fold with distilled water, by pipeting 50 ml. into a 500-ml. volumetric flask and diluting to the mark. This diluted solution is unstable and must be prepared daily.

0.5N Potassium iodide.—In a 500-ml. volumetric flask, 41.5 g. of potassium iodide is dissolved in distilled water, and the solution is diluted to the mark, stored in an actinic-red Pyrex bottle away from light, and discarded if any perceptible trace of iodine color develops.

Apparatus

A precision potentiometer capable of reading to ±0.1 mv. (for example, Leeds and Northrup type K-2 bridge) equipped with a sensitive galvanometer is needed. For less precise work, a pH meter with a millivolt scale may be used. Bright platinum and calomel electrodes are used. Also needed is a thermostated water bath controlled at 30.0° ± 0.1° and a stirring motor fitted with propeller-type glass stirrer.

Calibration of EMF Against Free Iodine

An accurate calibration chart must first be prepared to relate the EMF readings to the amount of free iodine in solution, under conditions identical with those employed in the starch titration. For this purpose, 373 mg. of potassium chloride and 830 mg. of potassium iodide are dissolved in 100.0 ml. of distilled water in a 250-ml beaker. The latter is placed in the 30° constant-temperature bath; the calomel and platinum electrodes are inserted, and the contents are stirred mechanically at a gentle rate with a glass propeller. Successive small amounts of the diluted iodine solution are added by buret, and the EMF is noted after each such addition, to cover in small increments the range of 230 to 285 mv. (Since the solution and the iodine reagent are both 0.05*N* with respect to potassium chloride and potassium iodide, there is no change in salt concentration during titration.) From the plotted curve of this titration a chart is prepared giving the milligrams of free iodine in solution corresponding to each millivolt reading from 230 to 260 mv. and to each half-millivolt reading from 260 to 285 mv. A new calibration chart should be prepared if either the potentiometer or the calomel electrode is changed.

Titration of Starch Samples

Before evaluation of iodine affinity, the starch sample must be thoroughly defatted by Soxhlet extraction for 24 hr. with 95% ethanol (Vol. IV [16]). The sample is then dried and pulverized to pass a 60-mesh screen. An appropriate amount (that is, approximately 40–50 mg. of linear frac-

tion, 200 mg. of branched fraction, or 100 mg. of whole starch) is weighed to an accuracy of ±0.1 mg. and transferred to a clean, dry 250-ml. beaker, previously tared to 0.1 g. on a sensitive torsion balance. Approximately 1 ml. of water is added to suspend the sample. Five ml. of 1.0*N* potassium hydroxide is added from a pipet, and the sample is dispersed in the alkali by grinding with a stirring rod, taking care to avoid the formation of insoluble lumps or clots. The mixture is placed in a refrigerator and allowed to stand 30 min. with occasional stirring, or until a perfectly clear solution is effected. It is then neutralized to methyl orange with 0.5*N* hydrochloric acid; 10 ml. of 0.5*N* potassium iodide solution is added, and the stirring rod is removed and rinsed into the beaker. Sufficient water is added to give a total weight of 100.9 g. over the weight of the empty beaker (that is, this represents the total addition of exactly 100.0 ml. of water at 30°). In a few instances (as with some unmodified granular starches), the sample will not dissolve to give the requisite crystal-clear solution in the alkali after standing 30–60 min. in the refrigerator. In such cases, the sample is neutralized, diluted with 25–50 ml. of water, and gently heated to assist solution. After cooling to room temperature, 10 ml. of 0.5*N* potassium iodide solution is added. Then the solution is neutralized and treated as above.

Except for the presence of the starchy substance, the resulting solution is identical with that used in the calibration. It is potentiometrically titrated with iodine at 30° with continuous mechanical agitation, the EMF being determined at some 10 to 15 points between 230 and 280 mv. Although the EMF stabilizes rapidly, it is advisable to wait 2 min. after each addition of iodine before balancing the potentiometer. For each point of the titration, the free iodine in solution is calculated from the corresponding EMF of the calibration chart, and this amount is deducted from the total amount of iodine added at that point (that is, ml. of iodine multiplied by 0.2000 mg.) to give the bound iodine. Free iodine is then plotted against bound iodine, as shown in Figure 1. The upper linear portion of this curve is extrapolated back to intersect the zero axis, and from this amount of bound iodine is calculated the iodine affinity of the sample. Results are converted to dry basis by separate moisture determination on a sample dried 4 hr. under reduced pressure at 120° (Vol. IV [10]):

$$\% \text{ Iodine affinity} = \frac{\text{mg. of bound iodine at zero intercept} \times 100}{\text{mg. of sample weight (on dry basis)}}$$

Precision of the method is such that replicate determinations should agree within ±0.08% iodine affinity. Typical values on the purified linear fractions from various starches (after repeated reprecipitation with 1-butanol) are as follows: corn, 19.0%; wheat, 19.9%; potato, 19.9%; tapioca,

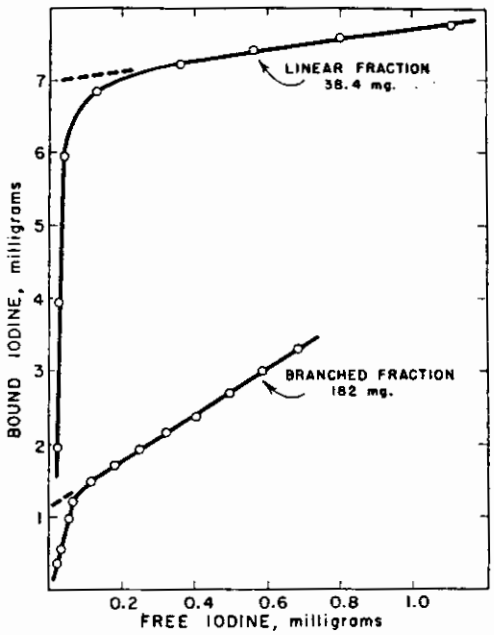
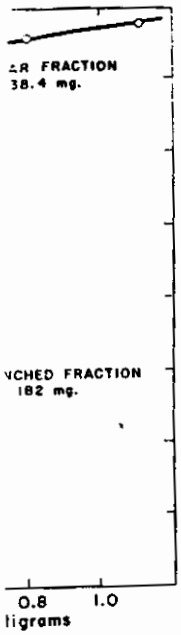


FIG. 1.—Graphical method for determination of iodine affinity by standard potentiometric titration. Sample size given in milligrams.

18.6%. It is strongly recommended that the linear content of starches be expressed merely as iodine affinity and not as percentage of amylose, since the status of possible intermediate fractions has not been clarified.

References

(1) F. L. Bates, D. French, and R. E. Rundle, *J. Am. Chem. Soc.*, **65**, 142 (1943).
(2) E. J. Wilson, Jr., T. J. Schoch, and C. S. Hudson, *J. Am. Chem. Soc.*, **65**, 1380 (1943).
(3) T. J. Schoch, "Methods in Enzymology," S. P. Colowick and N. O. Kaplan, eds., Academic Press Inc., New York, N.Y., Vol. 3, 1957, p. 13.



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S. P. Colowick and N. O. Kaplan,
., Vol. 3, 1957, p. 13.

[38] Iodimetric Determination of Amylose

Potentiometric Titration: Calcium Chloride Method for Modified Starches

By C. R. Colburn and Thomas J. Schoch

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Introduction

The standard iodine affinity method is not applicable to modified starches whose linear structure has been disturbed by oxidation, dextrinization, acid or enzyme hydrolysis, etherification or esterification. With such materials, the iodine titration curve does not have a sharp inflection point, and hence the plot of bound versus free iodine does not give a linear segment which can be extrapolated. The presently-described calcium chloride method[1] has been extensively used for such products and provides a reliable index of their relative linear character. While originally designed for commercial modified starches and dextrins, the method has likewise been satisfactorily used for the assay of linear material in a wide variety of natural starches (*1*), though incomplete solution and consequent low results were obtained with certain high-amylose starches. However, it should be noted that the calcium chloride method gives results quite different from the standard potentiometric titration (Table I).

Procedure

Special Reagents

Stock iodine solution.—166 g. of potassium iodide and exactly 4.000 g. of iodine are dissolved in a small amount of water in a 2-liter volumetric flask. When the iodine has completely dissolved, the solution is diluted to the mark and stored in a glass-stoppered brown glass bottle away from light. While reasonably stable, the solution should be periodically checked against standard sodium arsenite solution, and the factor corrected accordingly in calculations.

40° Baumé calcium chloride solution.—C.P. calcium chloride is dissolved in water to give a 40.0° Baumé solution at 30°, as determined by an accurate spindle calibrated in 0.1° Baumé units. Since calcium chloride

[1] The use of calcium chloride as a solvent for the starch and as a sequestrant for fatty acid was suggested by unpublished work of D. W. Howland and R. J. Smith, relating to spectrophotometric determination of blue color of starches with iodine.

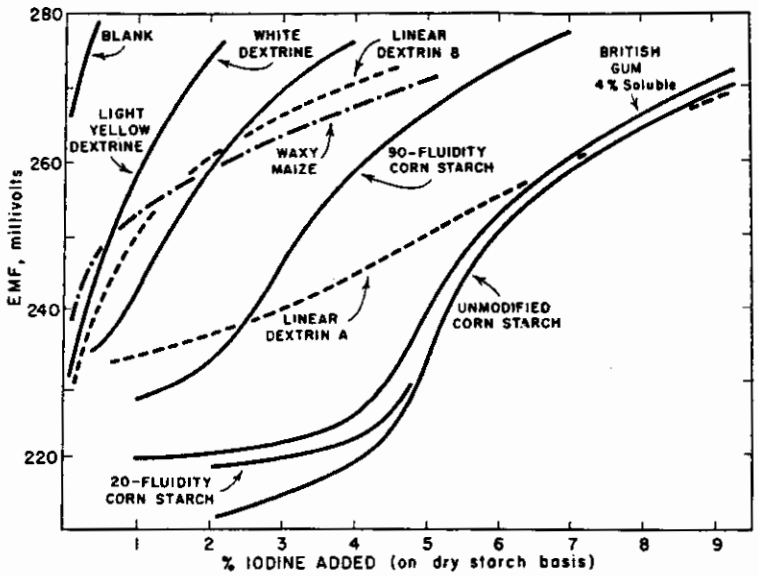


FIG. 1.—Complete iodine titration curves of various starch products in calcium chloride medium. Iodine affinity is calculated at the 240-mv. level. Linear dextrins A and B are short linear-chain products prepared by acid hydrolysis of pure corn linear fraction.

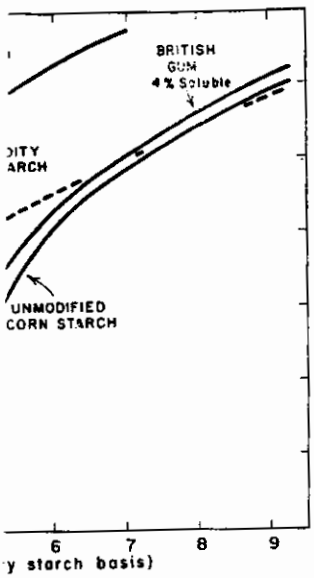
is usually slightly alkaline, the pH should be adjusted to 6.0 with dilute hydrochloric acid, using chlorphenol red or bromcresol purple as outside indicator. (The pH meter cannot be used.) The solution is suction-filtered through a pad of 4–5 filter papers on a Büchner funnel. It is advisable to run a blank titration on each batch of calcium chloride solution to ensure the absence of any iodine-reducing substances, that is, it should give an EMF above 265 mv. with a minimum amount of iodine solution, as shown in Figure 1.

Iodine solution for titration.—50.0 ml. of stock iodine solution is pipeted into a 500-ml. volumetric flask; 250 ml. of 40° Baumé calcium chloride solution is added, and the solution is diluted to the mark with distilled water. Final adjustment of volume should be made after thorough mixing and after equilibration in the 30° bath. This solution is unstable and should be prepared daily.

0.5N Potassium iodide solution is prepared and stored as described under the standard potentiometric titration (Vol. IV [37]).

Apparatus

As described for the standard potentiometric titration (Vol. IV [37]).



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Titration of Starch Samples

It is not necessary to ethanol-extract the sample for removal of natural fatty acids, since the calcium chloride completely sequesters fatty acid far in excess of the amounts naturally present in starch. However, if there is present any complexing agent for the linear fraction which does not form an insoluble calcium soap (for example, higher alcohols, monoglycerides, certain surfactants), the sample should first be Soxhlet-extracted for 24 hr. with 95% ethanol. To avoid lumping, the starch product should be ground to pass a 60-mesh screen. Moisture content is determined on a separate sample by drying for 4 hr. under reduced pressure at 120° (Vol. IV [10]).

A clean, dry 250-ml. beaker is tared to 0.1 g. on a sensitive torsion balance; 200 mg. of starch is weighed to the nearest 0.1 mg. and transferred to the beaker, and 5–10 ml. of water is added. (If the product is a pregelatinized starch which swells in cold water, 2–5 ml. of benzene is added instead of water to minimize formation of insoluble lumps.) Fifty ml. of 40° Baumé calcium chloride solution is added, meanwhile stirring with a glass rod to suspend the starch and to assist uniform gelatinization. The beaker is placed in a boiling water bath for 15 min., stirring continuously during the initial 2–3 min. of heating and occasionally thereafter. Then the beaker is cooled in cold water. The solution should be perfectly clear and homogeneous at this point; presence of any insoluble particles is due either to an improperly powdered sample or to insufficient stirring during the initial stages of gelatinization. A drop of methyl orange is added, and the solution is neutralized with 0.1*N* hydrochloric acid. The stirring rod is removed and rinsed into the beaker. The outside and the top rim of the beaker are dried, and the beaker is placed on the torsion balance. Water is added to give a total weight of 109.8 g. over the beaker tare. (After subsequent addition of 10 ml. of potassium iodide solution, this gives a final volume of 100.0 ml.) The beaker is placed in the 30° bath for 10 min.; the calomel and platinum electrodes are inserted, and 10 ml. of 0.5*N* potassium iodide solution is added. The resulting solution is titrated potentiometrically with the diluted iodine solution to 240 mv., with continuous mechanical stirring. As a preferred mode of titration, iodine is added until the EMF is between 230 and 235 mv. Additional small amounts of iodine solution are then added to give two readings between 235 and 240 mv. and two between 240 and 245 mv. The segment of the titration curve is then plotted for the four points (that is, ml. of iodine versus EMF), and the amount of iodine is interpolated for the 240-mv. point. This method of "straddling" the end-point is quicker and more precise than direct titration to 240 mv. It is advisable to wait 2 min. after each addition of iodine before reading the EMF. Adsorption of iodine by the starch is much more

TABLE I

Iodine Affinity of Various Modified Starches

Product	*% Iodine affinity*	
	$CaCl_2$ method	*Standard method*
Waxy maize starch, genetically pure	0.13	0
Light canary dextrin, 97% soluble	0.16	0
Dark yellow dextrin, 97% soluble	0.17	0
Light yellow dextrin, 98% soluble	0.29	0
Carboxymethyl starch, D.S. 0.5	0.60	0
White dextrin, 85% soluble	0.82	0
White dextrin, 65% soluble	0.87	0
Starch acetate, medium D.S.	1.17	4.7[a]
British gum, 80% soluble	1.27	0
White dextrin, 45% soluble	1.44	0
White dextrin, 25% soluble	1.77	0
Thin-boiling corn starch, 90-fluidity	2.71	1.5
White dextrin, 18% soluble	2.77	1.6
Starch acetate, low D.S.	2.80	5.1[a]
Brazilian tapioca starch, unmodified	3.69	—
Dominican tapioca starch, unmodified	3.72	3.3
White dextrin, 4% soluble	4.10	3.6
Hypochlorite-oxidized corn starch, 55-Scott viscosity	4.18	3.5
Hypochlorite-oxidized corn starch, 90-Scott viscosity	4.61	3.8
Maine potato starch, unmodified	4.97	4.1
Torrefaction gum, 4% soluble	5.05	4.1
Thin-boiling corn starch, 75-fluidity	5.22	4.2
Wheat starch, unmodified	5.25	—
Corn starch, unmodified, sample 1	5.31	—
Rice starch, unmodified	5.32	—
Thin-boiling corn starch, 40-fluidity	5.42	5.0
Corn starch, unmodified, sample 2	5.42	—
Thin-boiling corn starch, 60-fluidity	5.43	4.9
Thin-boiling corn starch, 20-fluidity	5.46	5.1
Wrinkled pea starch (var. "Laxton's Progress")	13.75[b]	15.0

[a] High results on starch acetates by standard method are due to deacetylation during solution in alkali, with consequent regeneration of non-derivatized starch.

[b] Incomplete solution by 15-min. cooking in calcium chloride.

complete in calcium chloride solution than in the potassium iodide–potassium chloride medium used in the standard method. Thus, a blank titration (Fig. 1) shows no free iodine up to 265 mv. Consequently, no correction is necessary for free iodine at 240 mv., and the per cent iodine affinity is directly calculated as:

$$\% \text{ Iodine affinity} = \frac{\text{ml. of iodine solution for 240 mv.} \times 0.200 \text{ mg. of iodine per ml.} \times 100}{\text{mg. of sample weight (on dry basis)}}$$

Replicate determinations should agree within ±0.02% iodine affinity. Certain variations in the procedure may be tolerated without affecting the final results, namely, the time of cooking may be varied from 10 to 30 min. and the sample size from 200 to 500 mg. However, it is essential that the temperature be maintained at exactly 30.0 ± 0.1° during the titration, since the iodine affinity decreases sharply with increase in temperature.

Table I lists iodine affinities of various starch products by the standard and the calcium chloride methods. The zero values cited for various modified starches by the standard method are due to excessive curvature of the plots for bound versus free iodine, thus preventing extrapolation to the zero axis. Figure 1 shows full titration curves of various starch products in calcium chloride medium. The 240-mv. endpoint was chosen experimentally as the inflection point most representative of various unmodified starches.

References

(1) W. L. Deatherage, M. M. MacMasters, and C. E. Rist, *Trans., Am. Assoc. Cereal Chemists*, **13**, 31 (1955).

[39] Iodimetric Determination of Amylose

Amperometric Titration

By J. N. BeMiller

Department of Chemistry, Southern Illinois University, Carbondale, Illinois

Introduction

The determination of the amount of iodine bound by the amylose in a starch and, hence, the determination of the amount of amylose in the starch can be accomplished by an amperometric adaptation of the dead-stop titration. The method described here is that of Larson and co-workers (*1*) in which iodine is generated in solution from iodide ions by reaction with iodate ions.

Reproducibility is not greatly affected by volume changes, changes in pH, changes in iodide concentration, or changes in amylose concentration

within the limits given. The end-point is sharp; the iodine uptake can be quickly calculated from a plotted curve, and the method uses potassium iodate as a primary standard, thus making it useful for rapid and routine determinations.

Holló and Szejtli (*2*) have used a similar dead-stop amperometric method with 0.005*N* iodine solution, which must be standardized frequently, as the titrant. Coton and co-workers (*3*) have used an amperometric titration to determine the absorption of iodine by suspensions of whole starch granules.

Procedure

Apparatus

Any standard polarographic instrument can be used to follow titrations. However, a highly precise voltage divider and meter are unnecessary and, as only relative current values are needed, calibration of the galvanometer can be omitted; thus, simpler equipment can be used. The apparatus described by Larson and Jenness (*1, 4*) is satisfactory and consists of a pair of No. 18 bright platinum wires about 4 cm. long and permanently mounted about 1 mm. apart as electrodes,[1] across which a potential of about 10 mv. is maintained, and a microammeter with a sensitivity of 0.2 μamp. or a galvanometer with low resistance and a sensitivity of 0.2 μamp./mm.

Starch Solutions

The starch sample is thoroughly defatted in a Soxhlet extractor for 24 hr. with 95% ethanol. The sample is then dried and pulverized to pass a 60-mesh screen. An appropriate amount (that is, 25–50 mg. of amylose, 100–200 mg. of whole starch, or 200–400 mg. of amylopectin) is weighed to an accuracy of ±0.1 mg. and transferred to a 100-ml. beaker. Approximately 1 ml. of water is added to suspend the sample. Ten ml. of *N* potassium hydroxide is added from a pipet, and the sample is dispersed in the alkali by grinding with a stirring rod, taking care to avoid the formation of insoluble lumps or clots. An additional 10.0 ml. of *N* potassium hydroxide solution is added, and the mixture is placed in a refrigerator for 30 min. (with occasional stirring) or until a perfectly clear solution is effected. This solution is then diluted with 20.0 ml. of water to give 40.0 ml. of starch solution in 0.5*N* potassium hydroxide.

[1] The electrodes must have a small surface area so that the time required for the current to reach equilibrium after a change in iodine concentration is short. However, the resistance of the electrode system (alternating current) must be low, or the deviation from linearity of the curve relating current flowing to free iodine concentration will be inconveniently large (*3*).

$$\% \text{ Iodine affinity} = \frac{\text{ml. of iodine solution for 240 mv.} \times 0.200 \text{ mg. of iodine per ml.} \times 100}{\text{mg. of sample weight (on dry basis)}}$$

Replicate determinations should agree within ±0.02% iodine affinity. Certain variations in the procedure may be tolerated without affecting the final results, namely, the time of cooking may be varied from 10 to 30 min. and the sample size from 200 to 500 mg. However, it is essential that the temperature be maintained at exactly 30.0 ± 0.1° during the titration, since the iodine affinity decreases sharply with increase in temperature.

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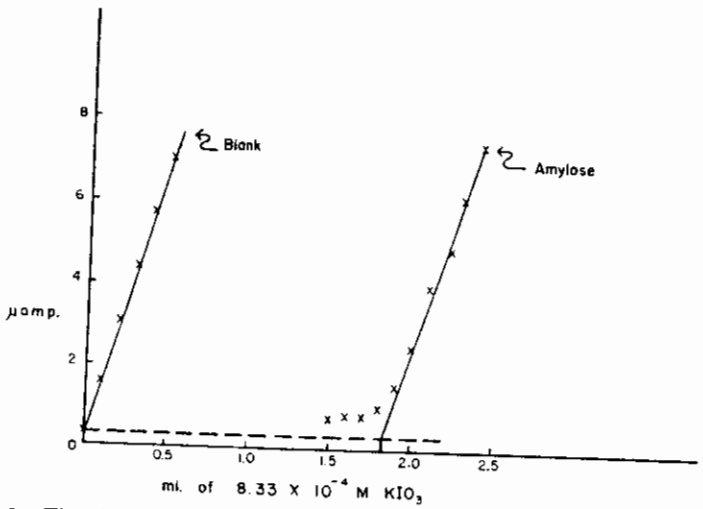

FIG. 1.—Titration curves for reagent blank and a 6.17-mg. sample of corn starch amylose [from Larson, Gilles, and Jenness (*1*)].

In some cases, the sample will not dissolve to give the requisite clear solution after standing 30–60 min. in the refrigerator. In such cases, the sample is gently heated with water to assist solution before the alkali is added, and the final volume is adjusted to make up that lost by evaporation.

Titration of Starch Samples

Into an extra high-form, 180-ml., electrolytic beaker, are introduced 10.0 ml. of the starch solution, 75 ml. of distilled water, 10 ml. of N hydrochloric acid, and 5 ml. of 0.4N potassium iodide solution. The beaker is set in a 25° constant temperature bath, and the mixture is stirred at a constant rate with a magnetic stirrer. An assembly carrying the electrodes and buret tip is lowered into the solution, and an initial reading is taken. Standard potassium iodate solution ($8.33 \times 10^{-4}M$) is added cautiously until a permanent galvanometer reading of about 1.5 μamp. is obtained. Increments of 0.1–0.2 ml. of the standard potassium iodate solution are then added with constant stirring, 1.5 min. being allowed before each galvanometer reading. The galvanometer readings are recorded until a current of about 8 μamp. is reached.

The end-point is obtained from these data by plotting ml. of $8.33 \times 10^{-4}M$ potassium iodate versus μamp. and extrapolating to the level of residual current flow where no iodate was added in the blank. A perpendicular dropped from this point to the abscissa reads directly in milliliters of iodate solution equivalent to the iodine that the sample has bound (Fig. 1).

mg. of iodine bound by 100 mg. of sample =

$$\frac{\text{ml. of } 8.33 \times 10^{-4}\ M\ KIO_3 \times 0.634}{\text{sample wt., mg.} \times 0.25}$$

% of amylose =

$$\frac{\text{mg. of iodine bound by 100 mg. of sample}}{\text{mg. of iodine bound by 100 mg. of "pure" amylose}} \times 100$$

in which the mg. of iodine bound by "pure" amylose is determined on a highly purified preparation of amylose (Vol. IV [7]) from the particular starch under analysis.

The entire procedure as described should take no longer than 10 min. It should be possible to use the method with a constant flow buret and a recorder if residual current is measured and flow from the buret in milliliters is calibrated against chart speed. However, the method is so simple and rapid that the author doubts if such an apparatus would save time, even for routine analyses.

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(1) B. L. Larson, K. A. Gilles, and R. Jenness, *Anal. Chem.*, **25**, 802 (1953).
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[40] Iodimetric Determination of Amylose

Iodine Sorption: "Blue Value"

By G. A. Gilbert and S. P. Spragg

Department of Chemistry, The University, Birmingham, England

Introduction

The absorbance of the blue color produced in aqueous solutions of amylose upon the addition of tri-iodide ion has long been used as a convenient indicator of the amount of linear fraction present in solution. The method was put on a quantitative basis by McCready and Hassid (*1*).

Procedure

To a 50-ml. flask is added 1.0 ml. of an aqueous solution containing approximately 0.5 mg. of linear fraction; 0.5 ml. of *N* sodium hydroxide is added, and the mixture is warmed 3 min. in a boiling water bath. After

mg. of iodine bound by 100 mg. of sample =

$$\frac{\text{ml. of } 8.33 \times 10^{-4}\ M\ KIO_3 \times 0.634}{\text{sample wt., mg.} \times 0.25}$$

% of amylose =

$$\frac{\text{mg. of iodine bound by 100 mg. of sample}}{\text{mg. of iodine bound by 100 mg. of "pure" amylose}} \times 100$$

in which the mg. of iodine bound by "pure" amylose is determined on a highly purified preparation of amylose (Vol. IV [7]) from the particular starch under analysis.

The entire procedure as described should take no longer than 10 min. It should be possible to use the method with a constant flow buret and a recorder if residual current is measured and flow from the buret in milliliters is calibrated against chart speed. However, the method is so simple and rapid that the author doubts if such an apparatus would save time, even for routine analyses.

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Procedure

To a 50-ml. flask is added 1.0 ml. of an aqueous solution containing approximately 0.5 mg. of linear fraction; 0.5 ml. of *N* sodium hydroxide is added, and the mixture is warmed 3 min. in a boiling water bath. After

cooling, an exactly equivalent amount of approximately N hydrochloric acid is added, followed by 0.07–0.1 g. of potassium hydrogen tartrate. Water is added to a volume of about 45 ml. and then 0.5 ml. of iodine solution (2 mg. of iodine/ml.; 20 mg. of potassium iodide/ml.). The solution is made up to 50 ml., mixed, and allowed to stand 20 min. at room temperature. The absorbance is measured at 680 mμ in a spectrophotometer using a 1-cm. cell. It is inadvisable to use an instrument which isolates the 680 mμ region by means of filters. For the reference solution, an iodine solution of equal concentration is used.

Usually the Beer–Lambert law is obeyed, but this should be confirmed. If so, then the "Blue Value" (B.V.) can be calculated from the equation:

$$\text{Blue Value} = \frac{\text{Absorbance} \times 4}{c(\text{mg./dl.})}$$

in which c refers to the carbohydrate content of the solution.

The problem of attaining a satisfactory solution of amylose is common to the whole of the chemistry of linear fractions. From this point of view, the drying of the linear fraction should be avoided if possible. If it is suspected that the blue color is not being fully developed because of retrogradation or incomplete solution, more vigorous methods of treatment should be adopted, and the method which gives the maximum absorbance should be chosen. The solution must always be approximately neutral before adding the buffering potassium hydrogen tartrate and iodine. If precipitation of amylose–iodide complex occurs before the measurement of absorbance, less amylose should be used or steps should be taken to reduce the total salt concentration. Where extracts from natural sources are involved, trouble may be experienced through chemical reaction with iodine. Appropriate precautions, for example, the addition of further iodine, must then be taken.

Reference

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[41] Determination of Amylose

Sorption of Congo Red

By Benjamin Carroll and Herbert C. Cheung[1]

Chemistry Department, Rutgers, The State University, Newark, New Jersey

Introduction

Congo red can be used for the colorimetric determination of amylose in starch. The method is based on the observation that the affinity of the dye for a carbohydrate substrate is markedly reduced by the presence of branching (*1–3*). The precision of this method is inferior to that of the colorimetric iodine method. This is due to the greater spectral change of the iodine indicator when sorbed by the substrate. However, precision in these determinations frequently may be misleading when properties of the Congo red and iodine indicators are compared. Two significant differences between the indicators are:

1. The spectral characteristics of *sorbed* Congo red are not altered by changes in the size or shape of the carbohydrate molecule.
2. The binding affinity of Congo red for amylose is independent of chain length over a wide range. Thus, it may be found that the Congo red method will yield an amylose content for an acid converted corn dextrin that is not much different from that for the original starch.

In view of the difference in behavior between Congo red and iodine, divergence in results by the two methods may be taken as a more adequate characterization of a starch sample. The effect of heating solid amylose (see Figure 1 and Table I) in terms of chain length and branching have been made clearer (*2*) by using both methods.

The effects of size of small branches on the dye affinity are not certain. Presumably there is a certain critical size below which the binding affinity is not significantly affected. Since the effect is not known, reference substances (amylose and amylopectin) of different botanical origins may lead to slightly different results on the amylose content of native starches.

Procedure

Preparation of Starch Solution

Starch solution is prepared by dissolving 0.1 g. (dry basis) of the starch in 5 ml. of standardized sodium hydroxide solution. A uniform solution may be obtained within 1 hr. The basic mixture is then neutralized with a

[1] Present Address: American Viscose Corporation, Marcus Hook, Pennsylvania.

cooling, an exactly equivalent amount of approximately N hydrochloric acid is added, followed by 0.07–0.1 g. of potassium hydrogen tartrate. Water is added to a volume of about 45 ml. and then 0.5 ml. of iodine solution (2 mg. of iodine/ml.; 20 mg. of potassium iodide/ml.). The solution is made up to 50 ml., mixed, and allowed to stand 20 min. at room temperature. The absorbance is measured at 680 mμ in a spectrophotometer using a 1-cm. cell. It is inadvisable to use an instrument which isolates the 680 mμ region by means of filters. For the reference solution, an iodine solution of equal concentration is used.

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Reference

(1) R. M. McCready and W. Z. Hassid, *J. Am. Chem. Soc.*, **65**, 1154 (1943).

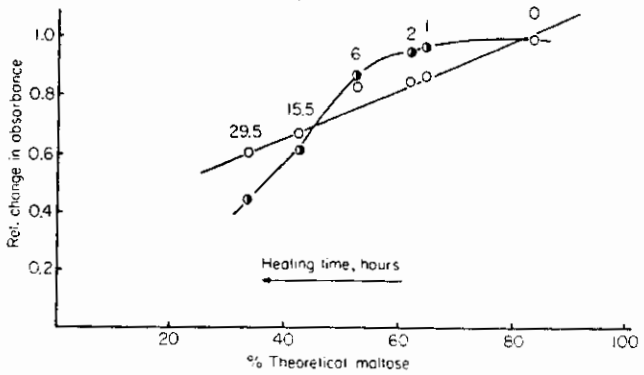

FIG. 1.—Effect of heating of granular amylose on the binding of Congo red and iodine. Relative change in absorbance versus per cent of theoretical maltose obtained upon complete hydrolysis of the heat-treated amylose with β-amylase. The numbers immediately above the points indicated the time of heating of the solid starch: ○○○ Congo red method; ◑◑◑ iodine method.

TABLE I

Amylose Content in Starch

Sample	*Iodine Affinity*[a]	*Amylose, %*	
		Colorimetric Iodine[b]	*Congo Red*[c]
Corn amylose	19.4	100.0[d]	100.0[d]
Washed 6 times with methanol, dried 1 hr. at 150°	18.7	96.0	73.0
Washed 6 times with methanol, dried 48 hr. at 150°	14.6	73.5	54.5
Corn amylopectin	0.8	0.0[d]	0.0[d]
Potato amylose	20.6	104.5	103.9
"Superlose" (potato amylose)	19.0	96.0	105.0
Defatted cornstarch, vacuum dried 1 hr. at 100°	5.2	24.5	20.0
"Dutch R-A" (potato starch)	3.7	29.5	27.0
High linear cornstarch	12.0	46.5	42.0
Wrinkled pea starch	15.0	76.5	70.0
"Ramalin G" (potato amylopectin)	1.1	10.0	0.3
Potato amylopectin	1.5	12.0	11.2
Washed 6 times with methanol, dried 1 hr. at 105°	0.8	10.1	9.9

[a] Milligrams of iodine taken up per 100 mg. of starch.

[b] The colorimetric iodine values obtained at 615 mμ.

[c] Congo red results obtained at starch concentration = 0.01%, pH = 4.2, temperature = 32°, and dye concentration = $2 \times 10^{-5} M$.

[d] Basis of calibration.

stoichiometric equivalent of standardized M hydrochloric acid solution and diluted to 100 ml. The neutralization should be done within 1.5 hr., and the neutralized solution should be used within 2 hr.

Colorimetric Measurements

Five ml. of pH 4.2 buffer (potassium hydrogen phthalate and sodium hydroxide) and 5 ml. of a 0.1% starch solution are introduced into a 50-ml. volumetric flask. Approximately 29 ml. of water is then added, followed by 10 ml. of $10^{-4}M$ Congo red solution (prepared from histological grade material). The resulting mixture is diluted to volume and the absorbance (A) at 500 mμ is measured immediately with a Beckman Model DU spectrophotometer using cells of 1-cm. path length. An appropriate starch solution is used as a blank. The final solution contains $2 \times 10^{-5}M$ Congo red and 0.01% starch.

Calculation of Amylose Content

Two standard substances are needed, one amylose and the other amylopectin (Vol. IV [7]). The binding of these two materials is determined as described above. The per cent of amylose of an unknown may be calculated from the following expression.

$$\% \text{ Amylose} = \frac{(A_s - A_{ap}) \times 100}{A_{am} - A_{ap}}$$

in which

A_s = absorbance of the sample–dye mixture
A_{ap} = absorbance of the reference amylopectin–dye mixture
A_{am} = absorbance of the reference amylose–dye mixture

Discussion

Because the Congo red method is of recent origin some comments are in order. Beer's law is followed by aqueous solutions of Congo red to within ±3%, up to a concentration of about $2 \times 10^{-5}M$ (3). The addition of starch to the dye causes the absorbance at the spectral peak to increase, as can be seen in Figure 2. Since the substrate concentration in each case is the same, the difference in absorbance among the curves may be assumed to be a function of the degree of branching. If the dye concentration is fixed, the absorptivity of the dye-starch mixture will increase with increasing starch concentration and reach a limiting value when the starch becomes in excess or all the dye ions are bound. The limiting value is found to be independent of the type and size of the substrate.

The linear relationship given in the above equation will be found to

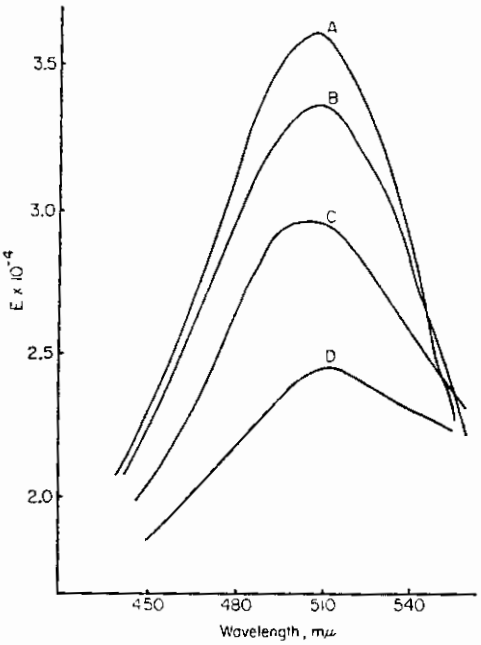

FIG. 2.—Spectral curves of aqueous Congo red in presence of starch and dextrin showing absorbance (E) × 10^{-4} versus wavelength. All solutions are buffered at pH 4.2, total substrate = 0.01%, dye = 2 × 10^{-5} M, temperature = 22°: A, "Superlose" (potato amylose); B, wrinkled pea starch (containing about 70% of amylose, see Table I); C, acid-converted dextrin; D, Congo red alone.

hold in the range of starch concentration specified in the procedure. Some results are shown in Table I and are compared with corresponding results from the usual potentiometric (Vol. IV [37]) and colorimetric (Vol. IV [40]) iodine methods. There appears to be a discrepancy between the Congo red and iodine results for the second and third samples listed. Both were corn amylose; one sample had been heated in the granular form for 1 hr. at 150° and the other for 48 hr. at 150°. The poor agreement is a reflection of different sensitivities of these two indicators toward branching and chain length.

In the procedure recommended, the sodium chloride concentration resulting from the preparation of the starch solution is approximately 0.005M. This concentration is not critical since the optical behavior of the dye itself if found to be independent of the salt concentration up to about 0.02M.

The choice of a pH of 4.2 is governed by the fact that spectral change is

optimal at this pH. This should be expected since the pK of the dye is about 4.1. It is to be noted that the pH should be carefully controlled when one is working in the region of the pK value.

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[42] Determination of Amylose Content of Corn Starch

Single Kernel Technique

By A. Cornwell Shuman and Richard A. Plunkett[1]

Shuman Chemical Laboratory, Inc., Battle Ground, Indiana

Introduction

This method determines linear fraction in corn endosperm directly, thus eliminating the need for preliminary isolation of starch. It is based largely on the method outlined by Kerr (*1*). A number of kernels can be analyzed to obtain an average value or a single kernel can be analyzed leaving the germ undamaged and available for subsequent embryo-culture.

Techniques are presented for analyzing the optimum number of samples (40) simultaneously. If single or small groups of samples are to be analyzed, appropriate equipment simplifications and procedure modifications can be made.

Procedure

Apparatus

Modified Polarimeter Tube.—A 2-dm. × 3.5-mm. (I.D.) polarimeter tube is modified to permit filling, emptying, rinsing, and drying with a water aspirator. Grooves are cut in opposite sides on each end of the tube and the brass end fittings to accommodate pieces of 18-gauge stainless steel tubing. The tubing is bent and placed so that its end is flush with the inside of the polarimeter tube and adjacent to the inside of the cover plate. Tubes and cover plates are cemented in place with epoxy resin. Polyethylene tubing [3/64 in. (1.2 mm.) I.D.] is attached to the stainless steel tubing.

[1] Present address: A. E. Staley Manufacturing Company, Decatur, Illinois.

Filtration Assembly.—Eight 150-mm. × 25-mm. Pyrex test tubes equipped with side arms are arranged in a rack; side arms are attached to an aspirator manifold with rubber tubing, and each tubing connection is fitted with a clamp so that the vacuum supply to each tube can be shut off when filtration is finished. These tubes hold 10-ml. screw-cap bottles or 15-ml. screw-cap centrifuge tubes and serve as filter flasks for filtration of the cooked samples.

Needle-tipped Pipets.—Half-inch lengths of 20-gauge stainless steel hypodermic needles are cemented into the tips of 1-ml. and 0.2-ml. graduated precision pipets. The needle tips contribute much to the precision of the pipeting operation because the amount of sample required to wet the tip is much less than that required with the conventional glass tip. The pipets are rinsed and dried with water and acetone using an aspirator.

Other Equipment.—Several other equipment items are required or facilitate multiple analyses: "Wig-L-Bug" grinder with stainless steel vials and balls; shaker; centrifuge; polarimeter, accurate to ±0.01°; spectrometer or spectrophotometer such as the Bausch and Lomb "Spectronic-20", modified as described by Creamer (*2*), and equipped with matching 11.66-mm. (0.5 in.) cuvettes; constant-temperature bath operating at 100°; a mixing wheel capable of rotating 100-ml. volumetric flasks end over end.

Reagents

Concentrated calcium chloride solution.—Reagent-grade calcium chloride dihydrate, 3.5 kg., is dissolved in 5 liters of water and the solution is suction filtered through a fine-porosity, fritted glass funnel. The specific gravity of the solution is adjusted to 1.30 (15°/15°) with water, and the pH adjusted to 2.0 ± 0.05 by dropwise addition of glacial acetic acid while stirring.

Dilute calcium chloride solution.—800 ml. of concentrated calcium chloride solution is diluted to 2 liters with water, and the resulting solution is thoroughly mixed.

Iodine solution, 1 mg. per ml.—Reagent-grade potassium iodide, 3.00 g. ± 0.01 g., is dissolved in about 25 ml. of water in a small flask. To this is added 2.100 g. ± 0.005 g. of reagent-grade iodine; the flask is covered loosely and warmed to about 40° to accelerate dissolution. When solution is complete, the contents are transferred quantitatively to a 2-liter volumetric flask and diluted to volume with water. The solution should be stored in an amber bottle and standardized by titration with 0.0500*N* standard thiosulfate. If concentration is not at least 0.0079*N*, it should be discarded and freshly prepared. The solution should be standardized weekly and discarded when the normality no longer conforms to the above value.

Mercuric chloride reagent, 0.1%.—1.0 g. of mercuric chloride is dissolved in about 100 ml. of water and diluted to 900 ml.; 100 ml. of ethanol is added, and the resulting solution is mixed.

Uranyl acetate solution.—10 g. of uranyl acetate dihydrate is dissolved in a mixture containing 80 ml. of water and 20 ml. of glacial acetic acid, heating if necessary but not above 60°; 100 ml. of the conc. calcium chloride solution is added, and the resulting solution is thoroughly mixed.

Method

The dissection of the grain is best effected on a glass plate which is easily cleaned between samples. The sample is obtained by clipping off a portion of the endosperm with a small, sharp pair of diagonal cutting pliers. With most grains this is readily done without injury to the germ when it is needed for subsequent embryo-culture. The sample pieces are cut or crushed so that no dimension is greater than ⅛ in. (3 mm.) and ground 2–3 min. in a "Wig-L-Bug" grinder using a stainless steel vial and ball. The sample is then passed through a 50-mesh sieve and thoroughly mixed. The ground sample should be stored in a freezer if not used immediately. Grinding is more efficient if the moisture content is about 14% or less.

The sample may contain 5 to 100 mg. of starch, but 20 to 40 mg. is the preferred range. This may be obtained from a single kernel or from a number of kernels. The sample is placed in a 15-ml. centrifuge tube and thoroughly wetted with a few drops of methanol to prevent clumping when the aqueous reagent is added. Ten ml. of the mercuric chloride reagent is added to inhibit enzymes; the tube is stoppered and shaken axially for 15 min. Up to forty samples can be shaken at one time by placing the tubes in a 40-place test tube rack, laying the rack on its side in the shaker and clamping it securely between two thick pieces of foamed plastic or rubber. Suspension of the sample is facilitated by including a 25-mm. × 2-mm. (diam.) stirring rod in each tube during the shaking. The tubes are than centrifuged until the precipitate is firm, the supernatant is decanted carefully. Ten ml. of dilute calcium chloride solution is added; the tubes are shaken and centrifuged as before, and the supernatant is decanted as completely as possible without losing the sample.

Concentrated calcium chloride solution (3.0–4.0 ml.) is added and the tube is shaken by hand to suspend the sample. The tube (with stopper removed) is placed in the heating bath and kept at 100° for 30 ± 3 min.; 1.0 ml. of uranyl acetate solution is added and mixed in by swirling. The resulting solution is decanted into a 30-ml. medium-porosity, fritted glass funnel containing a 3–5-mm. layer of Celite 545, and, with the aid of the filtration assembly, the filtrate is collected under reduced pressure in a 10-ml. screw-capped bottle. The filtrate must be sparkling clear; otherwise it should be re-filtered.

The optical rotation of the filtrate is measured in a 2-dm. tube, and the sample is saved for amylose measurement. Use of the modified polarimeter tube saves time when analyzing a series of samples because it can be filled, emptied, rinsed and dried without removing it from the polarimeter or touching it with the hands. Gentle suction applied to one end of the tube draws the sample into the tube without bubbles and without appreciable loss of sample. Release of the suction after reading the polarimeter permits the sample to return to the sample container by gravity flow. Then suction is used to rinse the tube with water and acetone, and to dry the tube with air. These operations are done in the dark room permitting the operator to make measurements on a series of samples while his eyes are adapted to the dark.

A volume of sample containing about 1 mg. of amylose (usually 0.1–1.0 ml.) is pipeted into a 100-ml. volumetric flask containing about 90 ml. of dilute calcium chloride solution. The volume of sample is chosen to give an absorbance of 0.20–0.45 for highest accuracy. Under the conditions of this method, the absorbance per mg. of amylose is 0.33–0.35, and that of amylopectin is 0.02–0.05. Iodine solution (4.0 ml.) is added by pipet, and the flask is filled to the mark with dilute calcium chloride solution. The solution absorbance must be measured within 2–5 min. after mixing to achieve consistent results. It is necessary to wait at least 2 min. after mixing for maximum color development since the amylose–iodine complex is formed slowly. It is then important to measure quickly because the complex will soon begin to agglomerate and precipitate, most quickly with high-amylose samples and almost immediately in the case of pure amylose.

When analyzing 40 samples at a time, the above requirements are best met by a semi-production line method. Forty 100-ml. volumetric flasks are prepared with 90 ml. of dilute calcium chloride solution, and the proper amount of sample is added to each. A reagent blank is prepared by diluting 4.0 ml. of iodine solution to 100 ml. with dilute calcium chloride solution. The flask containing the blank is clipped to a mixing wheel and mixed. Iodine solution is added to the first sample, and the mixture is diluted to volume. The first sample is clipped to the mixing wheel, and the reagent blank is removed. The spectrometer is adjusted to read 0.00 absorbance at 600 mμ with the reagent blank in the 11.66-mm. (nominally 0.5-in.) cuvette. Then the cuvette is emptied and rinsed. Iodine is added to the second sample, and this mixture diluted to the mark. The second sample is clipped to the mixing wheel, and the first is removed and read. Succeeding samples are diluted, mixed, and read in turn. Using these techniques, one sample can be read every 2–3 min. which is about optimum time. The cuvette should be rinsed twice with portions of the first sample, before the absorbance is measured. Between samples, one rinsing is usually

sufficient. The spectrometer adjustments should be rechecked every 0.5 hr. with a freshly-prepared reagent blank.

Calculations

Starch content of the calcium chloride solution extract of the sample is calculated from the observed optical rotation.

$$c = \text{Starch Content of Extract (mg. per ml.)} = \frac{\alpha \times 1000}{203^\circ \times 2 \text{ dm.}}$$

in which, α = observed angular rotation in degrees, 2 dm. = length of polarimeter tube, 203° = specific rotation of corn starch.

Absorbance of the starch–iodine complex is expressed as an absorption coefficient, k = absorbance per mg. of starch, which is related to the amylose content of the starch.

$$k = \frac{\text{Observed sample absorbance}}{\text{Sample aliquot, ml.} \times c}$$

$$\% \text{ Amylose (starch basis)} = 322k - 18.8$$

in which, 322 and 18.8 are calibration constants.

Calibration and Precision

The method is calibrated against the potentiometric titration method described by Bates and co-workers (*3*) and modified by Schoch and co-workers (*4*). The equation, $y = 322k - 18.8$, in which y is % amylose by the potentiometric method and k is the absorption coefficient (absorbancy per mg.) of the starch–iodine complex, represents the regression line calculated from results obtained on sixteen samples. Amylose contents of samples ranged from that in waxy corn starch to butanol-precipitated amylose. The coefficient of correlation was 0.959.

Analysis of variance of duplicate analyses on eighteen samples of corn representing a wide range of amylose content—from waxy to high-amylose starch—gave a standard error of $0.0054k$ or 1.73% amylose, and 95% confidence limits of $\pm$ $0.0108k$ $\pm$ 3.46% amylose for a single determination.

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